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Genetic evidence for involvement of type 1, type 2 and type 3 inositol 1,4,5-trisphosphate receptors in signal transduction through the B-cell antigen receptor

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Stimulation of B-cell antigen receptor (BCR) induces a rapid increase in cytoplasmic free calcium due to its release from intracellular stores and influx from the extracellular environment. Inositol 1,4,5-trisphosphate receptors (IP_3Rs) are ligand-gated channels that release intracellular calcium stores in response to the second messenger, inositol 1,4,5-trisphosphate. Most hematopoietic cells, including B cells, express at least two of the three different types of IP_3R. We demonstrate here that B cells in which a single type of IP_3R has been deleted still mobilize calcium in response to BCR stimulation, whereas this calcium mobilization is abrogated in B cells lacking all three types of IP_3R. Calcium mobilization by a transfected G protein-coupled receptor (muscarinic M1 receptor) was also abolished in only triple-deficient cells. Capacitative Ca^{2+} entry, stimulated by thapsigargin, remains unaffected by loss of all three types of IP_3R. These data establish that IP_3Rs are essential and functionally redundant mediators for both BCR- and muscarinic receptor-induced calcium mobilization, but not for thapsigargin-induced Ca^{2+} influx. We further show that the BCR-induced apoptosis is significantly inhibited by loss of all three types of IP_3R, suggesting an important role for Ca^{2+} in the process of apoptosis.

Keywords: apoptosis/B-cell antigen receptor/calcium mobilization/inositol 1,4,5-trisphosphate receptor/muscarinic receptor

Introduction

Stimulating the B-cell antigen receptor (BCR) initiates a cascade of signal transduction events in which cytoplasmic protein tyrosine kinase (PTK) activation is the earliest known event. At least three types of cytoplasmic PTKs, Src-PTK, Syk and Btk, are responsible for the initiation of BCR-induced signals (Pleiman *et al.*, 1994; Weiss and Littman, 1994; Bolen, 1995; DeFranco, 1995). These intracellular signaling events are coordinated to lead to a variety of biological responses, depending on the developmental stage of B cells (Rajewsky, 1996). Mature B lymphocytes undergo proliferation and antibody production in response to BCR cross-linking, whereas immature B lymphocytes die by an apoptotic process.

BCR-induced tyrosine phosphorylation of phospholipase C (PLC)-γ2, which is mediated by Syk and Btk, is responsible for its increased activity, allowing the conversion of phosphatidylinositol 4,5-bisphosphate into the second messenger inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Rigley *et al.*, 1989; Takata *et al.*, 1994; Takata and Kurosaki, 1996). DAG activates protein kinase C (PKC) (Nishizuka, 1988), and IP_3 is thought to cause Ca^{2+} release from intracellular stores after binding to its receptor.

Several DNA-binding proteins, notably NF-κB, AP-1 and CREB, are induced after B-cell activation (Chiles *et al.*, 1991; Liu *et al.*, 1991; Rooney *et al.*, 1991; Chiles and Rothstein, 1992; Lalmanach-Girard *et al.*, 1993; Xie *et al.*, 1993). These factors are induced by cross-linking of BCR or by treatment with phorbol ester (PMA) alone, suggesting that they are activated via the PKC pathway. However, PMA alone is not sufficient to trigger B-cell activation, which requires an additional calcium signal. One of the DNA-binding proteins which is regulated via the calcium signal is NF-AT. NF-AT is composed of at least two components (Flangan *et al.*, 1991): a nuclear component (NF-ATn) that is synthesized *de novo* in response to PKC or Ras activation, and a pre-existing cytoplasmic subunit (NF-ATp, NF-ATc, NF-AT3, NF-AT4/x) that is translocated to the nucleus (Boise *et al.*, 1993; Castigli *et al.*, 1993; Jain *et al.*, 1993; Northrop *et al.*, 1993, 1994; Hoey *et al.*, 1995; Masuda *et al.*, 1995). Strong evidence suggests that the effect of increased $[Ca^{2+}]_i$ on NF-ATc translocation is mediated by the action of the calcium/calmodulin-dependent phosphatase calcineurin (reviewed by Schreiber and Crabtree, 1992). Although NF-AT was described initially as an inducible T cell-specific DNA-binding protein that is required for interleukin-2 (IL-2) gene expression, recent studies have shown that NF-AT is also activated upon BCR cross-linking (Choi *et al.*, 1994; Venkataraman *et al.*, 1994), suggesting it to be a more general nuclear factor.

IP_3R is a Ca^{2+} channel localized on the endoplasmic reticulum (ER) membrane. Electron microscopic observations (Chadwick *et al.*, 1990; Maeda *et al.*, 1990), cross-linking data (Maeda *et al.*, 1991) and sucrose gradient centrifiguration experiments have demonstrated that IP_3Rs form tetramers (Mignery *et al.*, 1989). To date, three types of IP_3R, derived from three distinct genes, have been discriminated (Furuichi *et al.*, 1989; Mignery *et al.*, 1990; Südohf *et al.*, 1991; Ross *et al.*, 1992; Yoshikawa *et al.*, 1992; Blondel *et al.*, 1993; Kume *et al.*, 1993; Maranto, 1994). These three types of IP_3R are co-expressed in a variety of hematopoietic cells and lymphocyte cell lines (Sugiyama *et al.*, 1994; Yamamoto-Hino *et al.*, 1994). Analyses of the three types of IP_3R cDNA sequences

have shown overall structural organization in three basic domains: an amino-terminal IP_3-binding domain, a carboxy-terminal Ca^{2+} channel domain and a linking domain containing sites for regulatory processes. Type 2 IP_3R has significant homology with the type 1 IP_3R (total 69%), especially in the IP_3-binding and Ca^{2+} channel domains. Type 3 shares 64% overall amino acid homology with type 1 IP_3R (reviewed by Furuichi *et al.*, 1994). The channel domain is sufficient for assembly of the subunits to yield the tetrameric organization of the IP_3R (Mignery and Südohf, 1990; Miyawaki *et al.*, 1991). Since this domain is well conserved among members of IP_3Rs, the possibility of the formation of heterotetramers has been proposed (Monkawa *et al.*, 1995).

Recent antisense studies have shown functional distinctions between type 1 and type 3 IP_3Rs in T cells. Transfection with type 3 IP_3R antisense constructs, but not type 1 antisense, prevented dexamethasone-induced increases in $[Ca^{2+}]_i$, leading to blockade of apoptosis (Khan *et al.*, 1996). Together with the evidence that only type 3 IP_3R is augmentally localized on the plasma membrane upon dexamethasone treatment, these data provide the possibility that type 3 IP_3R specifically participates in Ca^{2+} entry through the plasma membrane, leading to apoptosis. In contrast to this report, Jurkat T cells in which type 1 antisense constructs were expressed failed to show increased $[Ca^{2+}]_i$ and produce IL-2 after T-cell antigen receptor (TCR) stimulation, showing the importance of type 1 IP_3R (Jayaraman *et al.*, 1995). Thus, an exact functional dissection among IP_3R subtypes still remains elusive. Moreover, since a sphingosine kinase pathway rather than the IP_3 pathway through PLC activation has been demonstrated to mediate FcεRI-induced Ca^{2+} mobilization in mast cells (Choi *et al.*, 1996), it is not completely clear whether the BCR-induced calcium mobilization is dependent on the IP_3R(s).

To test directly the functional roles of each IP_3R, we established mutant B-cell lines that lack various combinations of the three types of IP_3R. Here we show that BCR-induced calcium mobilization is only abrogated in B cells lacking all three types of IP_3R, demonstrating the functional redundancy among these IP_3Rs. Similarly, calcium mobilization by a transfected G protein-coupled receptor was abolished only in triple-deficient cells, indicating that both receptors utilize IP_3Rs for calcium mobilization. In contrast, capacitative Ca^{2+} entry, stimulated by thapsigargin (TG), remained intact even in the triple-deficient cells. Moreover, BCR-induced apoptosis was significantly inhibited by the loss of all three types of IP_3R. Since this apoptosis is abolished completely in PLC-γ2-deficient mutant B cells, this result suggests that both the calcium and PKC pathway are required for BCR-induced apoptosis.

Results

Targeted disruption of IP_3Rs

As seen in Figure 2A, RNA blot analysis revealed that type 1, type 2 and type 3 IP_3Rs were all expressed in the DT40 B-cell line. For disruption of the type 1 IP_3R locus, mutations of the two type 1 IP_3R alleles were introduced into DT40 cells by sequential homologous recombinations (Figure 1A). The targeting vectors contain a histidinol or hygromycin resistance gene cassette replacing the chicken genomic sequence, which contains exons corresponding to the channel region. Similarly, disruption of the type 2 or type 3 IP_3R locus was carried out (Figure 1B and C). To disrupt both type 1 and type 2 IP_3R loci, we transfected type 2 IP_3R targeting constructs containing puromycin and blasticidin resistance gene cassettes into type 1 IP_3R-deficient DT40 cells. Furthermore, type 3 IP_3R targeting constructs containing bleomycin and mycophenolic acid resistance gene cassettes were transfected sequentially into type 1/type 2 double-deficient cells to isolate triple IP_3R-deficient DT40 cells (Figure 1). Homologous recombination events were screened by Southern blot analysis using the probes shown in Figure 1, and at least two independent clones were identified. Each targeting vector was incorporated as a single copy, as revealed by Southern analysis using probes of each drug resistance cassette. To verify null mutations, Northern blot analyses using specific probes of each type of IP_3R gene were carried out. As shown in Figure 2A, mutant cells failed to express RNA of the corresponding type of IP_3Rs. The level of cell surface expression of BCR on various IP_3R-deficient clones was essentially the same as that of parental DT40 cells (Figure 2B).

IP_3Rs are utilized redundantly for BCR- and muscarinic M1 receptor-induced calcium mobilization

Stimulation of the BCR induced a rapid $[Ca^{2+}]_i$ increase in wild-type DT40 cells. EGTA incubation reduced the peak height of this $[Ca^{2+}]_i$ increase ~2-fold (data not shown), suggesting that some fraction of the $[Ca^{2+}]_i$ increase is derived from intracellular pools. DT40 cells lacking only type 1, type 2 or type 3 IP_3Rs exhibited a $[Ca^{2+}]_i$ increase. In contrast to these single IP_3R-deficient cells, no increase in $[Ca^{2+}]_i$ was detected in DT40 cells lacking all three types of IP_3R (Figure 3). IP_3 production of these deficient DT40 cells upon BCR stimulation was essentially the same as that of wild-type cells (Figure 4). These results provide direct evidence that IP_3Rs are essential for BCR-induced calcium mobilization from intracellular pools as well as calcium influx, and suggest that the three types of IP_3R participate in BCR-induced calcium mobilization in a redundant manner. DT40 cells lacking both type 1 and type 2 IP_3Rs still showed calcium mobilization upon BCR cross-linking, although the amplitude of the BCR-induced $[Ca^{2+}]_i$ increase was decreased reproducibly ~2-fold compared with wild-type cells (Figure 3). The peak height of this calcium mobilization was also inhibited ~2-fold by treatment with EGTA (data not shown), suggesting that the $[Ca^{2+}]_i$ increase is derived from both release from intracellular pools and influx from the extracellular environment. Since these double-deficient B cells express type 3 IP_3Rs, these data indicate that the type 3 IP_3R is capable of inducing a $[Ca^{2+}]_i$ increase in the absence of type 1 and type 2 IP_3Rs. On the contrary, as shown above, the absence of only type 3 IP_3R did not affect the BCR-induced calcium mobilization, further supporting the functional redundancy among the three types of IP_3R.

To address whether a G protein-coupled receptor utilizes IP_3R for mobilizing calcium, we transfected the M1 muscarinic receptor into wild-type and various IP_3R-

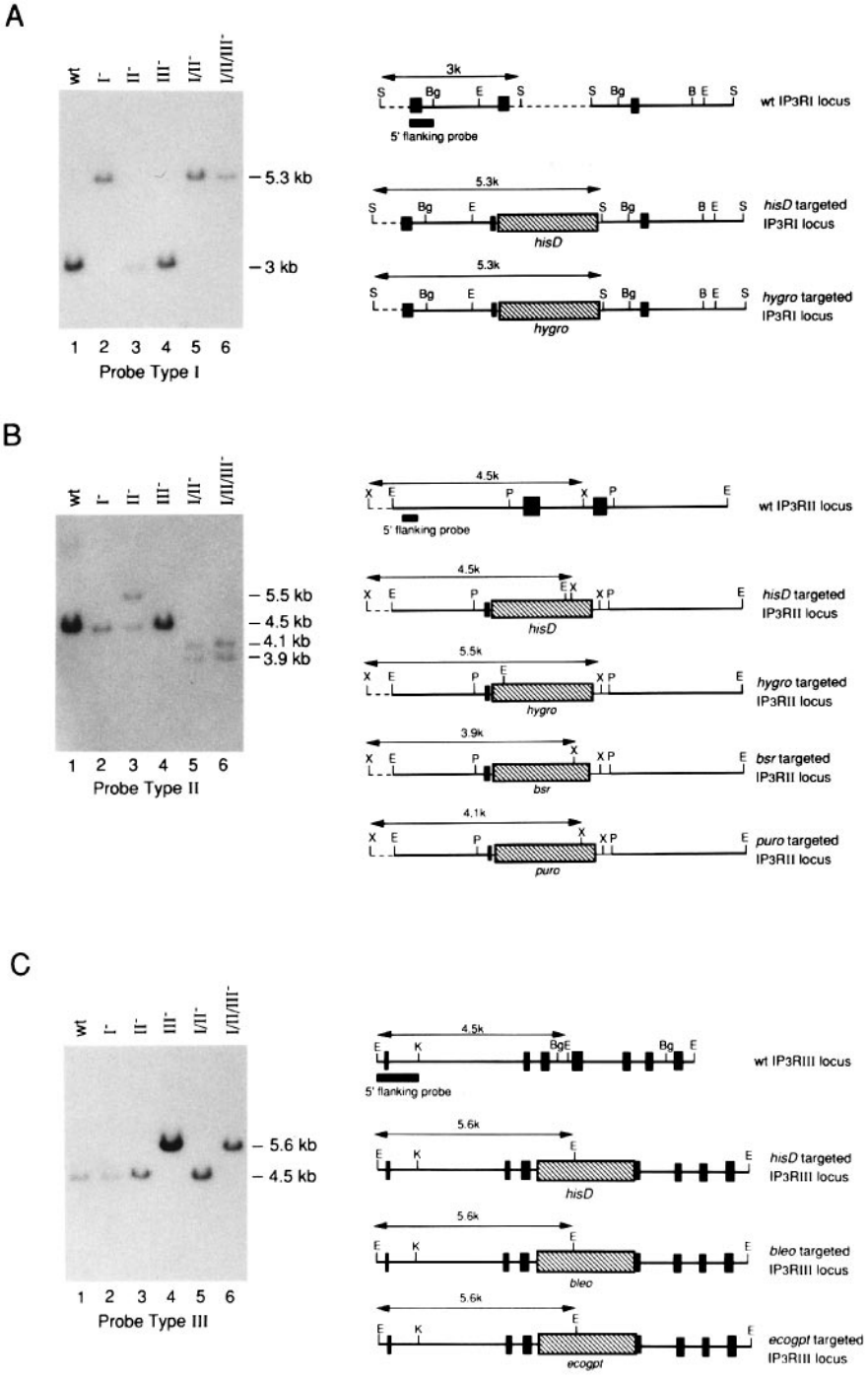

Fig. 1. Targeted mutation of the IP_3R genes. Targeted mutation of type 1 (**A**), type 2 (**B**) and type 3 (**C**) IP_3R genes. Southern blotting of various mutant clones (left side) and the expected structure of the disrupted alleles (right side) are shown. Genomic DNAs were prepared, digested with *Sac*I (type 1), *Xba*I (type 2) and *Eco*RI (type 3), and hybridized with each 5′-flanking probe. Although the type 2 IP_3R wild-type allele and the *hisD*-targeted allele showed the same size 4.5 kb band by *Xba*I digestion (lane 3 in B), we confirmed that this 4.5 kb was derived from the targeted allele by genomic Southern blotting with *Eco*RI digestion. The restriction endonuclease cleavage sites are abbreviated as B = *Bam*HI; Bg = *Bgl*II; E = *Eco*RI; K = *Kpn*I; P = *Pst*I; S = *Sac*I; X = *Xba*I.

deficient cells. Transformants showing similar [^{3}H]-quinuclidinyl benzilate ([^{3}H]QNB) binding in various IP_3R-deficient cells were isolated and characterized. The M1 muscarinic receptor is known to evoke IP_3 generation through G protein-coupled PLC-β activation by agonist stimulation (Berridge, 1993). Indeed, these transformants showed IP_3 production upon stimulation of the muscarinic receptor agonist, carbachol (data not shown). As shown in Figure 3, the carbachol-induced increase in $[Ca^{2+}]_i$ was still observed in DT40 cells deficient in only one type of IP_3R or type 1/type 2 double-deficient cells, whereas this calcium mobilization could not be detected in triple-deficient DT40 cells. Similarly to the BCR-induced calcium mobilization in type 1/type 2 double-deficient cells, the amplitude of the carbachol-induced $[Ca^{2+}]_i$ increase was ~3-fold lower than that of wild-type cells. Since the

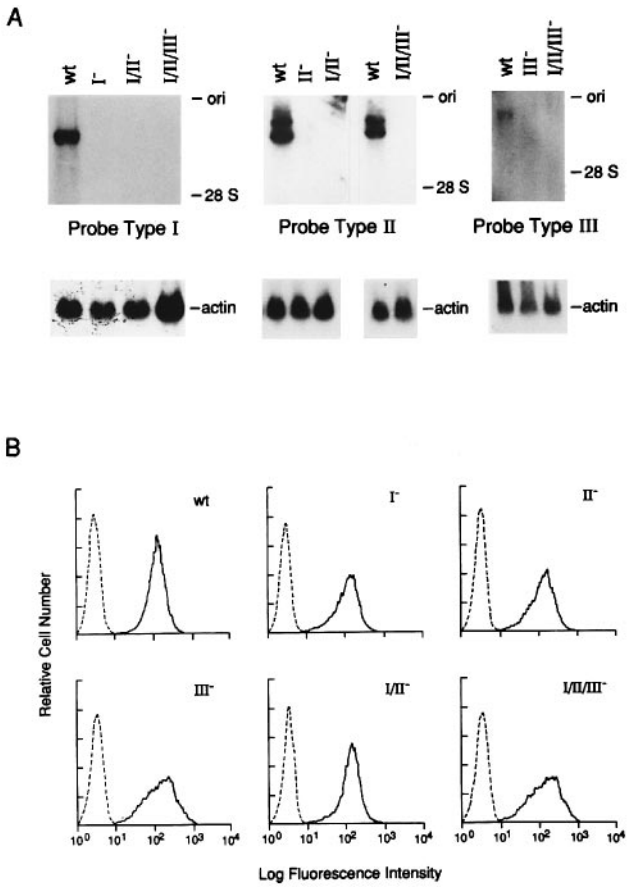

Fig. 2. RNA expression analysis of various IP_3R mutant DT40 cells (**A**) and BCR expression on various mutants (**B**). RNA (20 μg) was separated in a 1.2% formaldehyde gel, blotted and probed with IP_3R type-specific probes (similar size). The periods of exposure for the autoradiographs were 14 (type 1 IP_3R), 100 (type 2 IP_3R) and 150 h (type 3 IP_3R). The positions of the origin and 28S are shown in (A). DT40 cells were stained with FITC-conjugated anti-chicken IgM Ab in (B). Unstained cells were used as negative controls (dotted lines).

level of cell surface expression of M1 on the double-deficient cells was the same as that of wild-type cells, this low amplitude of $[Ca^{2+}]_i$ was not due to the expression level of M1. These results suggest that the BCR and M1 muscarinic receptor utilize similar mechanisms to mobilize calcium after production of IP_3.

Thapsigargin-induced Ca^{2+} influx is not affected by loss of the three types of IP_3R

To determine whether IP_3R(s) in B cells participates directly in calcium influx, we used thapsigargin (TG) to deplete calcium from intracellular stores. TG is an inhibitor of the ER Ca^{2+}-ATPase. By blocking Ca^{2+} uptake, TG unmasks a constitutive leak of Ca^{2+} from the ER and thereby depletes intracellular stores (Guoy *et al.*, 1990; Thastrup *et al.*, 1990; Lytton *et al.*, 1991; Mason *et al.*, 1991). Ca^{2+} store depletion is known to trigger Ca^{2+} influx through plasma membrane Ca^{2+} channels by a process referred to a capacitative Ca^{2+} entry (Berridge, 1993; Putney and Bird, 1993). Similarly to wild-type DT40 cells, treatment of cells lacking various combinations of IP_3R with TG resulted in a sustained increase in $[Ca^{2+}]_i$ (Figure 3). Using wild-type and triple-deficient DT40 cells, we further examined the characteristics of Ca^{2+} influx after Ca^{2+} readdition to Ca^{2+}-depleted cells. In these experiments, the Ca^{2+} stores were first maximally depleted by adding TG to cells in the absence of extracellular Ca^{2+}. In both wild-type and mutant cells, subsequent addition of media containing 2 mM Ca^{2+} evoked a substantial $[Ca^{2+}]_i$ increase due to influx through depletion-activated Ca^{2+} channels in the plasma membrane (Figure 5). These results show that IP_3Rs are not involved in TG-induced capacitative Ca^{2+} entry.

NF-AT activity is defective in DT40 cells lacking all three types of IP_3R

DT40 cells lacking all three IP_3Rs have allowed us to dissect the PKC and Ca^{2+} pathways in BCR signaling. For this purpose, we first sought to confirm whether PKC activation is still intact in DT40 cells lacking all three types of IP_3R. Since MARCKS protein is well known to be a physiological substrate of PKC (Aderem, 1992), we measured induction of phosphorylation of MARCKS protein upon BCR stimulation. As expected, wild-type DT40 cells exhibited ~3-fold stimulation of phosphorylation of MARCKS protein, whereas this stimulation was abolished completely in DT40 cells deficient in PLC-γ2 (Takata *et al.*, 1995). In cells lacking all three types of IP_3R, this induction was still observed (Figure 6), indicating that DAG, not Ca^{2+} through IP_3Rs, is critical for PKC activation upon BCR cross-linking.

Some transcription factors such as NF-AT are reported to be induced after BCR stimulation (Choi *et al.*, 1994; Venkataraman *et al.*, 1994). To determine the effects of Ca^{2+} on NF-AT under physiological conditions, we transfected a reporter gene containing NF-AT-binding sites and the luciferase gene into mutant cells. Whereas NF-AT-directed transcription was readily apparent in wild-type DT40 cells upon BCR ligation, it was undetectable in PLC-γ2-deficient cells (Takata *et al.*, 1995). In cells deficient in all three types of IP_3R, NF-AT-dependent transcription was abrogated (Figure 7). The BCR-induced NF-AT activity was observed in type 1/type 2 double-deficient DT40 cells, although the extent of stimulation was two-thirds of that in wild-type cells. These results indicate that the BCR-induced increase in $[Ca^{2+}]_i$ is essential for NF-AT activity.

Ca^{2+} is required for BCR-induced apoptosis

We have shown previously that BCR-induced apoptosis is almost completely abolished in PLC-γ2-deficient DT40 cells (Takata *et al.*, 1995), suggesting that the PKC pathway and/or Ca^{2+} pathway are required for apoptosis. Thus, to clarify the involvement of the Ca^{2+} pathway in BCR-induced apoptosis, we used DT40 cells lacking various combinations of IP_3Rs. Treatment of wild-type DT40 cells with monoclonal antibody (mAb) M4 resulted in a drastic increase in the percentage of apoptotic cells, as assessed by propidium iodide staining and flow cytometric analysis, whereas in PLC-γ2-deficient cells apoptosis was almost completely abolished (Figure 8). IP_3R-deficient cells exhibited a reduction in apoptosis which was intermediate; double-deficient cells exhibited an ~1.5-fold reduction, and cells lacking all three types of IP_3R exhibited an ~2.5-fold reduction in BCR-induced apoptosis. This inhibition was restored by addition of ionomycin to M4 stimulation (data not shown). These

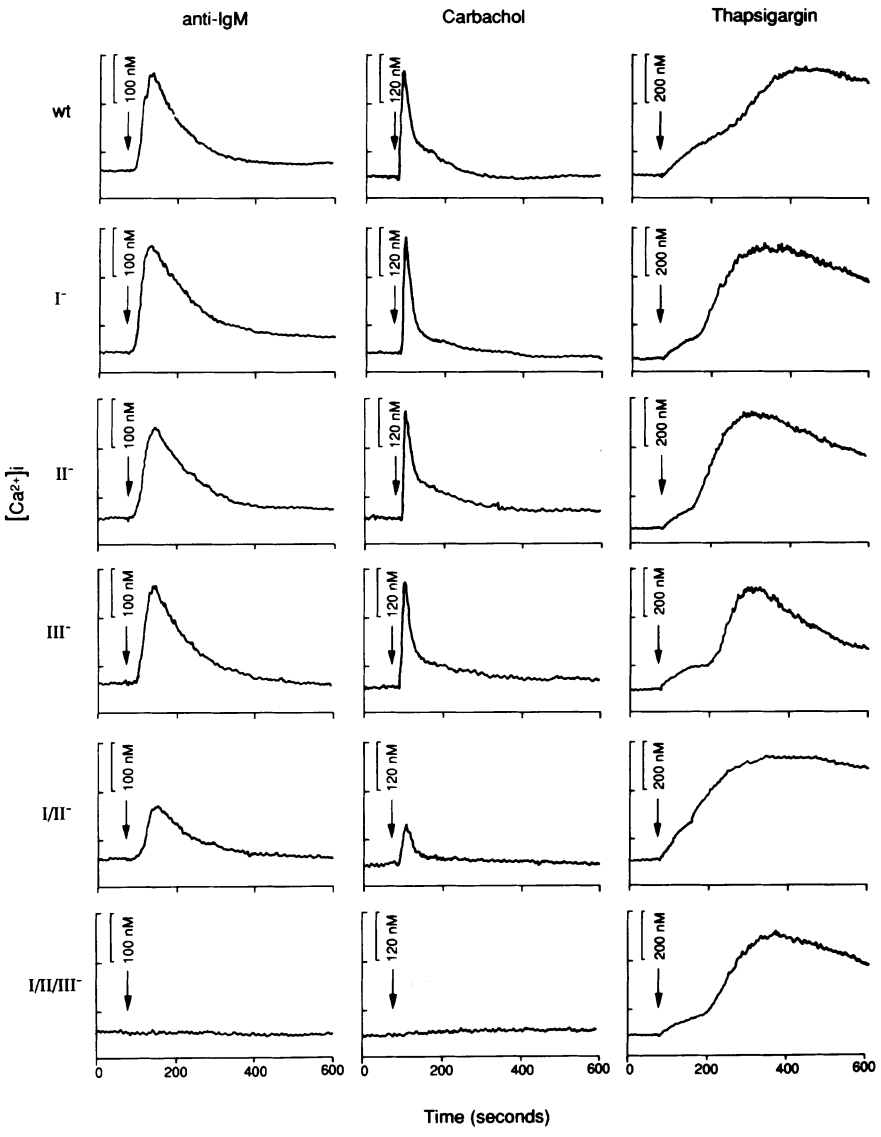

Fig. 3. BCR-induced and muscarinic M1-induced calcium mobilization in various combinations of disruptions in the IP_3R genes. Various mutant cells were stimulated with anti-BCR mAb M4 (2 μg/ml), carbachol (500 μM) and thapsigargin (2 μM) (added at the arrow).

results show that both PKC activation and calcium mobilization are required for BCR-induced apoptosis. Because the BCR-induced apoptosis was not affected by loss of only one type of IP_3R (data not shown), and double-deficient cells showed the intermediate level of apoptosis between wild-type and triple-deficient cells, this calcium requirement for apoptosis appears to be dependent on the $[Ca^{2+}]_i$ level upon BCR stimulation.

Discussion

Cells have at least two intracellular channels for regulating calcium release from internal stores; ryanodine receptors (Berridge, 1993; Clapham, 1995), first discovered in muscle but now known to exist in other cell types including lymphocytes (Hakamata *et al.*, 1994), and IP_3Rs. In addition, it was proposed recently that sphingosine-1-phosphate mediates FcεRI-induced calcium mobilization, presumably through its receptor (Choi *et al.*, 1996). Thus, it is not clear which intracellular channel system is

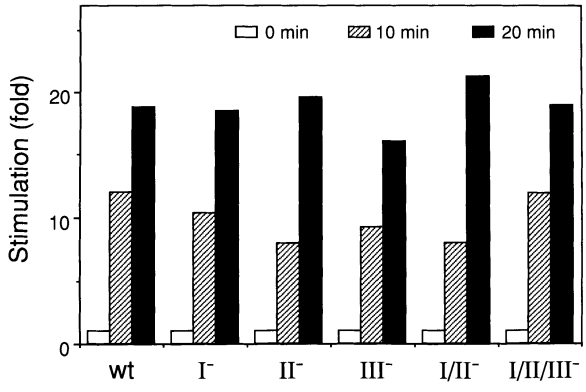

Fig. 4. BCR-induced IP_3 generation of various IP_3R mutant DT40 cells. Cells were stimulated with anti-BCR mAb M4 (2 μg/ml). After 10 or 20 min of stimulation, soluble inositol phosphates were extracted and separated by AG 1-X8 ion exchange columns.

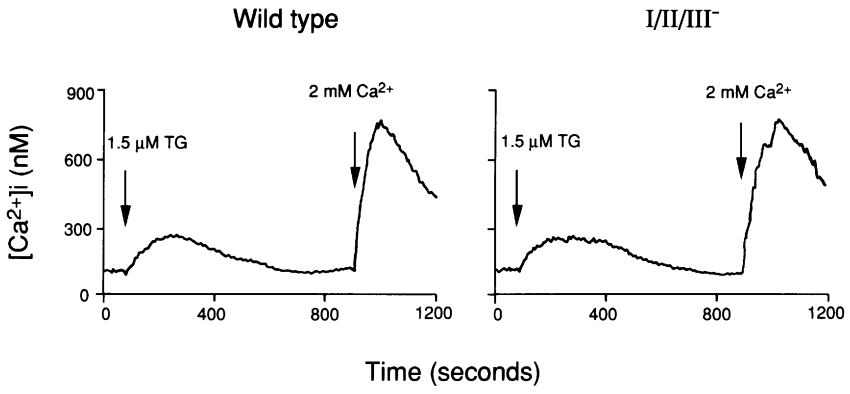

Fig. 5. Thapsigargin (TG)-induced calcium entry was intact in DT40 cells lacking all three types of IP_3R. Intracellular Ca^{2+} release and Ca^{2+} influx were measured in TG-stimulated wild-type and mutant cells. After washing of cells with Ca^{2+}-free Ringer's, 1.5 μM TG was used to deplete the Ca^{2+} stores, followed by addition of 2 mM Ca^{2+}.

principally involved in BCR-induced calcium mobilization.

Here we have used mutant DT40 cells deficient in various combinations of IP_3Rs to address this issue. Analyses of these mutant cells established that the loss of a single type of IP_3R had no effect on BCR-induced calcium mobilization, whereas the $[Ca^{2+}]_i$ increase was abolished completely in triple-deficient DT40 cells (Figure 3). These data provide direct evidence that the IP_3R system is essential for the increase in $[Ca^{2+}]_i$ upon BCR cross-linking and that the three different IP_3Rs can exhibit functional redundancy in BCR signaling. The mutant cells which exhibit a reduced $[Ca^{2+}]_i$ by virtue of expressing only type 3 IP_3R represent an interesting case. A number of possibilities may account for the diminution in $[Ca^{2+}]_i$ exhibited by loss of type 1 and type 2 IP_3Rs. This may reflect lower abundance of type 3 IP_3R compared with other types of receptors in DT40 cells. This hypothesis may be supported by the reduced band intensity seen in Northern analysis (Figure 2A), where the level of type 3 transcript was ~20- and ~5-fold lower than type 1 and type 2, respectively. Another possibility is that type 3 IP_3R may have lower functional activity, such as IP_3-binding activity or channel activity, compared with other types of IP_3R. It is also possible that heterotetramers among IP_3Rs (Mignery and Südhof, 1990; Miyawaki *et al.*, 1991; Monkawa *et al.*, 1995), for instance between type 1 and type 3, are required for full functional activity. Neverthless, our results clearly indicate that the type 3 IP_3R alone, to a certain extent, is able to mobilize calcium from both inside and outside the cells in response to BCR stimulation.

Previous studies have shown that Jurkat T cells, which like DT40 cells express all three types of IP_3R (Sugiyama *et al.*, 1994), fail to exhibit an increase in $[Ca^{2+}]_i$ after TCR stimulation when type 1 antisense constructs are expressed (Jayaraman *et al.*, 1995). These data are in contrast to our results; however, as Jayarman *et al.* mentioned in their report, the antisense construct used in their studies may also cross-react with type 2 IP_3R and weakly with type 3 IP_3R, thereby potentially leading to an inhibition of all three types of IP_3R. It is also possible that the Jurkat cells transfected with antisense constructs lose type 2 and type 3 IP_3Rs during drug selection. Obviously, the distinct usage of IP_3R subtypes by TCR and BCR is a most intriguing possibility, and deserves further study.

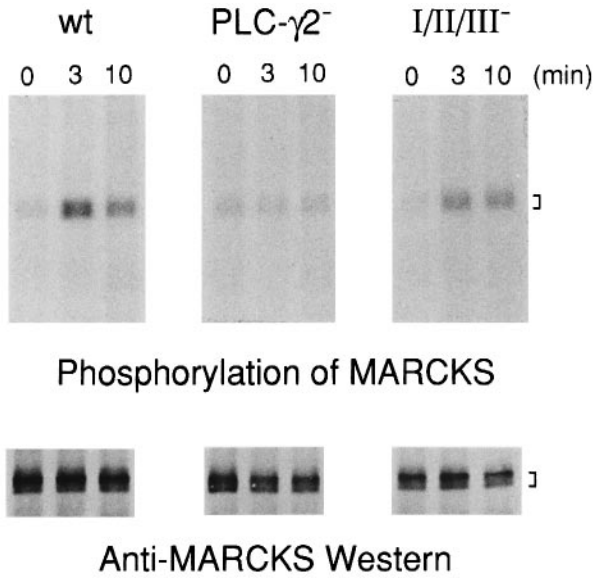

Fig. 6. PKC activation in wild-type, PLC-γ2- and all three types of IP_3R-deficient DT40 cells. Cells were labeled with [^{32}P]orthophosphate, stimulated with mAb M4 (2 μg/ml) and immunoprecipitated by anti-MARCKS antibody. Immunoprecipitates were electrophoresed on an 8% SDS–PAGE gel and autoradiographed. Half of the immunoprecipitates were used for Western blotting (lower panel).

In DT40 cells, Ca^{2+} mobilization can be activated by either of two cell surface receptors: the endogenous BCR, signaling through PLC-γ2 activated by Syk and Btk (Takata *et al.*, 1994; Takata and Kurosaki, 1996), or the stably transfected M1 muscarinic receptor that signals through a G protein-regulated PLC-β (Berridge, 1993). The biochemical signaling through these two receptors is quite dissimilar, but both lead to the production of IP_3 and result in calcium mobilization. Compared with the BCR-induced increase of $[Ca^{2+}]_i$, carbachol-induced calcium release is substantially rapid (Figure 3). This probably reflects the rapid kinetics of the G protein-linked signaling cascade that couples the M1 receptor to IP_3 production. M1-induced calcium mobilization was eliminated completely only in triple IP_3R-deficient DT40 cells, and loss of both type 1 and type 2 IP_3Rs significantly inhibited

the $[Ca^{2+}]_i$ increase upon carbachol stimulation. These calcium mobilization profiles induced by stimulation of the stably transfected M1 receptor in various DT40 mutant cells show noteworthy similarities to those induced upon BCR cross-linking, suggesting that both receptors utilize similar mechanisms to mobilize calcium after production of IP_3.

It has been proposed that FcεRI-coupled calcium mobilization is mediated by a sphingosine kinase (SK) pathway rather than the IP_3 pathway (Choi *et al.*, 1996). In the rat mast cell line RBL-2H3, in contrast to IP_3 production upon stimulation of a transfected M1 receptor, FcεRI-mediated IP_3 production is substantially less. Instead, stimulation of FcεRI produces sphingosine-1-phosphate through SK activation, thereby leading to calcium mobilization. Since both FcεRI and BCR transmit their signals through similar biochemical mechanisms, such as through PTKs, Lyn and Syk (Kurosaki *et al.*, 1994; Takata *et al.*, 1994; Scharenberg *et al.*, 1995), these data might raise questions regarding our conclusion that both the BCR and M1 receptor utilize the IP_3 pathway in DT40 cells. However, in DT40 cells, stimulation of BCR evoked the same or a higher level of IP_3 production than stimulation of the M1 receptor (data not shown). Thus, a simple explanation is that since the level of IP_3 upon FcεRI stimulation is not sufficient to activate IP_3Rs, a back-up mechanism through the SK pathway might operate in mast cells. Such a redundant back-up mechnism already has been shown between ryanodine and IP_3 receptor systems. Ryanodine and IP_3 receptors, through binding of cyclic ADP-ribose (cADPR) (Galione *et al.*, 1991, 1993a; Mészáros *et al.*, 1993) and IP_3 respectively, contribute to the fertilization calcium wave in sea urchin eggs. Inhibition of either pathway had no effect, but the fertilization wave was abolished when both messengers were knocked out (Galione *et al.*, 1993b; Lee *et al.*, 1993). The cADPR seems to be much more restricted to certain cell types than is IP_3. Similarly, the messenger function of shingosine-1-phosphate may be more restricted to certain cell types. For instance, mast cells might utilize both the IP_3- and sphingosine-1-phosphate-dependent pathways, whereas only the IP_3-dependent pathway is available in B cells. Other potential explanations for the signaling differences between the BCR and FcεRI may exist, such as the inability of B cells to produce sphingosine-1-phosphate via activation of SK, despite similar biochemical signal transduction mechanisms for both receptors.

Rapid calcium mobilization following BCR stimulation comprises two phases: the initial phase consists of a transient release of calcium from intracellular stores and is followed by a sustained calcium influx caused by the opening of the calcium channel present in the plasma membrane. Our current data support the capacitative model

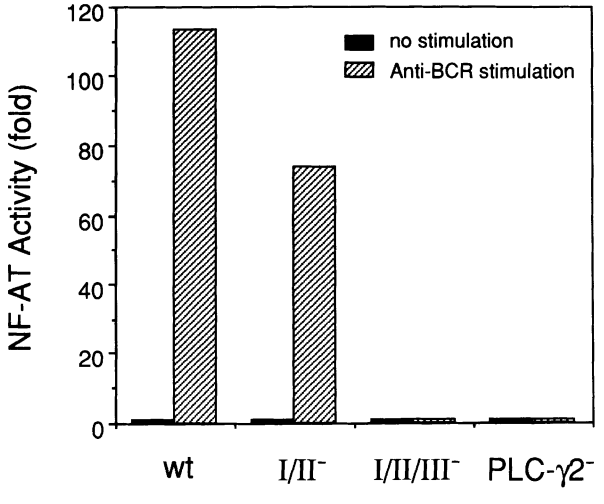

Fig. 7. NF-AT activity in various mutant cells. Cells transfected with the NF-AT luciferase gene were analyzed as described in Materials and methods. Twenty four hours following elcectroporation, cells were divided and treated in the absence or presence of anti-BCR mAb (M4, 3 μg/ml). Transfection efficiency was normalized using pRL-CMV. The experiment shown is representative of three independent trials.

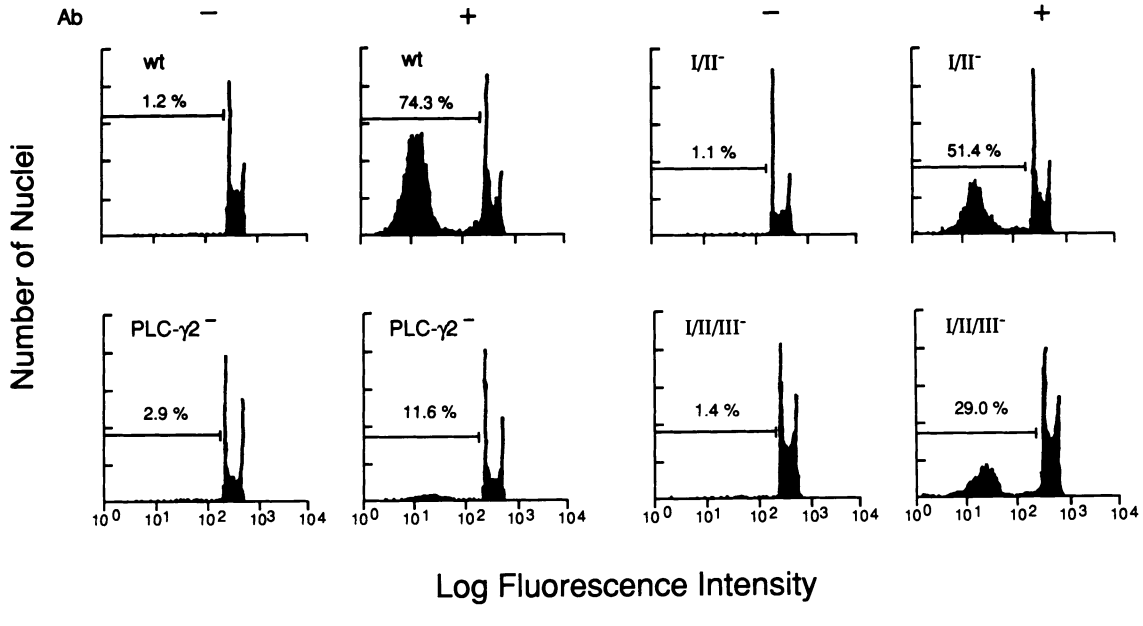

Fig. 8. Induction of apoptosis in various mutant DT40 cells. Cells were cultured with (+) or without (–) mAb M4 (10 μg/ml, 24 h), treated in hypotonic DNA staining solution containing 50 μg/ml propidium iodide and subjected to analysis by FACScan.

for Ca^{2+} entry in which Ca^{2+} influx across the plasma membrane is coupled to depletion of intracellular Ca^{2+} stores (Putney and Bird, 1993; Berridge, 1995; Clapham, 1995). The mechanism by which the depletion of intracellular Ca^{2+} stores leads to plasma membrane Ca^{2+} influx has not been clearly established. It has been suggested that a conformational change in the IP_3R, induced by emptying of the ER Ca^{2+} pool, may lead to the opening of a plasma membrane Ca^{2+} channel (Berridge, 1995). Assuming that the TG-induced Ca^{2+} channel corresponds exactly to this channel, our data show that capacitative Ca^{2+} entry is still intact in cells lacking all three types of IP_3R (Figure 5) and argue against a role for the type 1, type 2 and type 3 IP_3Rs in communicating between the ER and the plasma membrane channel as proposed in the conformational coupling hypothesis. A large variety of mediators between the ER and the plasma membrane channel have been proposed, including a novel diffusible messenger (Parekh *et al.*, 1993; Randrimampita and Tsien, 1993) and small GTP-binding proteins (Bird and Putney, 1993; Fasolato *et al.*, 1993). The present data indicate that IP_3Rs are not the target for such mediators.

The involvement of type 3 IP_3R, not type 1, in Ca^{2+} entry has been proposed, based on the data on the inhibition of dexamethasone-induced calcium mobilization using antisense approaches (Khan *et al.*, 1996). Since our data show normal BCR-induced calcium mobilization in type 3 IP_3R-deficient DT40 cells (Figure 3), the general involvement of type 3 IP_3R in calcium influx seems to be unlikely. However, our data do not exclude the possibility that the type 3 IP_3R participates in specifically dexamethasone-induced calcium entry.

Recent studies show that a factor, indistinguishable from T-cell NF-AT, is induced in B cells in response to BCR signaling (Choi *et al.*, 1994; Venkataraman *et al.*, 1994). Similarly to the requirement for both PKC and Ca^{2+} for NF-AT activity in T cells, it has been demonstrated that in normal B cells the NF-AT activity is induced in response to the combined action of phorbol ester and ionomycin, but not in response to either reagent alone. Our triple IP_3R-deficient DT40 cells have allowed us to dissect the requirement of PKC and Ca^{2+} for induction of NF-AT activity using a genetic rather than a pharmacological approach. As expected, these triple-deficient B cells were still able to induce PKC activity, as assessed by phosphorylation of MARCK protein (Figure 6), whereas NF-AT activity was abrogated completely in the triple IP_3R-deficient DT40 cells (Figure 7). This further strengthens the notion that Ca^{2+} is essential for NF-AT activity upon BCR stimulation. This Ca^{2+} effect is presumably through the action of calcium/calmodulin-dependent phosphatase calcineurin, since suppression of BCR-induced NF-AT activity by cyclosporin has been demonstrated previously (Choi *et al.*, 1994; Venkataraman *et al.*, 1994).

Most self-reactive immature B cells are eliminated during development by negative selection (clonal deletion) to establish immunological self-tolerance. This process of clonal deletion is thought to be mediated by apoptosis (Schwartz, 1989; Goodnow, 1992; Nossal, 1994). Many of the signaling processes during apoptosis of B cells have been studied using transformed B-cell lines (Rothstein, 1996). When stimulated with anti-BCR antibodies, DT40 B cells undergo apoptosis. This BCR-induced apoptosis was suppressed significantly in triple IP_3R-deficient DT40 cells. However, in contrast to the almost complete inhibition of apoptosis in PLC-γ2-deficient DT40 cells (Takata *et al.*, 1995), residual apoptosis remains in the triple-deficient cells (Figure 8), suggesting that both the PKC and the Ca^{2+} pathway are required for this cell death. Thus, B-cell lines lacking PLC-γ2 and the three types of IP_3R will provide the tools to further our understanding of the mechanisms by which BCR-induced apoptosis is regulated through PKC and Ca^{2+}.

Materials and methods

Cells, expression vectors and antisera

DT40 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin and glutamine. To construct chicken expression vector pAneo, a *Hin*dIII–*Cla*I 0.7 kb fragment containing the puromycin resistance gene was replaced with a *Hin*dIII–*Cla*I 1.2 kb fragment containing the neomycin resistance gene from the pBabeNeo vector (Morgenstern and Land, 1990). Murine MARCKS cDNA (Seykora *et al.*, 1991) and porcine muscarinic M1 receptor cDNA (Kubo *et al.*, 1986) were cloned into the pApuro (Takata *et al.*, 1994) and the pAneo vectors. These cDNAs were transfected by electroporation using Gene pulser apparatus (Bio-Rad Laboratories) at 550 V, 25 μF, and selected in the presence of either 0.5 μg/ml puromycin or 2 mg/ml G418. Expression of transfected cDNA was confirmed by Western blot analysis (MARCKS) or binding assay (M1 muscarinic receptor). Anti-chicken IgM mAb M4 and antisera against the murine MARCKS were described previously (Chen *et al.*, 1982; Seykora *et al.*, 1991).

Generation of IP_3R-deficient DT40 cells

The chicken spleen cDNA and genomic DNA libraries were obtained from Clontech. The chicken cDNA library was screened by the mouse type 1 IP_3R cDNA (kindly provided by Drs Furuichi and Mikoshiba) (Furuichi *et al.*, 1989) under low stringency condition. Several chicken cDNA clones were sequenced to confirm the identification of type 1, type 2 and type 3 chicken IP_3R genes. To isolate chicken genomic clones of type 1, type 2 and type 3 IP_3R genes, the chicken genomic library was screened by each type-specific chicken cDNA probe. After subcloning the genomic clones of chicken type 1, type 2 and type 3 IP_3R genes, the targeting constructs were made. The *hisD*, *hygro*, *ecogpt* and *bsr* cassettes for these constructs were described previously (Hartman and Mulligan, 1988; Takeda *et al.*, 1992; Takata and Kurosaki, 1996). The drug resistance genes for *puro* and *bleo* cassettes were derived from pBabePuro and pBabeBleo (Morgenstern and Land, 1990), respectively.

The targeting vectors of the type 1 IP_3R gene were constructed by replacing the genomic sequence, which contains exons corresponding to amino acid residues 2531–2630 of human type 1 IP_3R (Harnick *et al.*, 1995), with a *hisD* or *hygro* cassette (pIP_3R type 1-hisD or -hygro, respectively). The upstream 1.5 kb genomic sequence was generated by PCR using the type 1 genomic clone as a template and the downstream sequence was derived from a 3.0 kb *Sac*I–*Sac*I genomic fragment. The targeting vectors of the type 2 IP_3R gene were constructed by replacing the 2.9 kb genomic sequence, which contains exons corresponding to amino acid residues 2415–2469 of human type 2 IP_3R (Yamamoto-Hino *et al.*, 1994), with a *hisD*, *hygro*, *bsr* or *puro* cassette (pIP_3R type 2 -hisD, -hygro, -bsr or -puro, respectively). The upstream 2.3 kb genomic sequence was generated by PCR using the type 2 genomic clone as a template and the downstream sequence was derived from a 3.5 kb *Pst*I–*Eco*RI genomic fragment. The targeting vectors of the type 3 IP_3R gene were constructed by replacing the 0.8 kb genomic sequence, which contains exons corresponding to amino acid residues 2191–2217 of human type 3 IP_3R (Yamamoto-Hino *et al.*, 1994), with a *hisD*, *bleo* or *ecogpt* cassette (pIP_3R type 3-hisD, -bleo or -ecogpt, respectively). The upstream genomic sequence was derived from a *Kpn*I–*Eco*RI 3.4 kb fragment and the downstream 2.4 kb genomic sequence was generated by PCR using the type 3 genomic clone as a template.

The targeting vectors were linearized and transfected into DT40 cells by electroporation (550 V, 25 μF). After isolation of several clones in the presence of various drugs (1 mg/ml histidinol, 2 mg/ml hygromycin, 50 μg/ml blasticidin S, 0.5 μg/ml puromycin, 0.3 mg/ml phleomycin and 30 μg/ml mycophenolic acid), genomic DNAs were prepared and analyzed by Southern blot analysis. For isolation of single disruptions

of each IP_3R gene, two targeting constructs were transfected sequentially into wild-type DT40 cells (pIP_3R type 1-hisD and -hygro for disruption of the type 1 IP_3R gene, pIP_3R type 2-hisD and -hygro for disruption of the type 2 IP_3R gene, and pIP_3R type 3-hisD and -bleo for disruption of the type 3 IP_3R gene).

For isolation of type 1/type 2 double-deficient cells, pIP_3R type 2-bsr and -puro were transfected sequentially into type 1 IP_3R-deficient cells. For isolation of triple-deficient cells, pIP_3R type 3-bleo and -ecogpt were transfected sequentially into the type 1/type 2 double-deficient DT40 cells.

Cell surface expression of BCR was analyzed by FACScan using FITC-labeled anti-chicken IgM. A single clone of each targeted mutant was analyzed extensively, although some critical experiments were carried out using at least two different clones.

Northern blot analysis

RNA was prepared from wild-type and mutant DT40 cells using the guanidium thiocyanate method. Total RNA (20 μg) was separated in a 1.2% formaldehyde gel, transferred to Hybond-N membrane (Amersham) and probed with ^{32}P-labeled cDNAs. Probes used were cDNA fragments specific for each type of chicken IP_3R gene and the chicken β-actin gene (Kost *et al.*, 1983).

Calcium analysis

Measurements of intracellular free calcium levels were performed with fura-2/AM. Cells (5×10^6/ml) were washed once and loaded with 3 μM fura-2/AM in phosphate-buffered saline (PBS) containing 20 mM HEPES (pH 7.2), 5 mM glucose, 0.025% bovine serum albumin (BSA) and 1 mM $CaCl_2$. After 45 min of incubation at 37°C, cells were washed twice and diluted to 10^6 cells/ml with the same buffer. Fluorescenece of the stirred cell suspension was monitored continuously with a fluorescence spectrophotometer Hitachi F-2000 at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. $[Ca^{2+}]_i$ was calibrated and computed as described (Grynkiewicz *et al.*, 1985). Using two independent clones from each IP_3R-deficient mutant, calcium measurements were carried out three times for each clone (total $n = 6$). In the case of type1/type2 double-deficient cells, three different clones were used for this analysis (total $n = 9$). To chelate extracellular calcium, 3 mM EGTA was added for 1 min before stimulation.

Phosphoinositide analysis

Cells (10^6/ml) were labeled with myo-[^{3}H]inositol (10 μCi/ml, Amersham) for 6 h in inositol-free RPMI 1640 supplemented with 10% dialyzed FCS. The cells (5×10^6/ml) were pre-equilibrated at 37°C and stimulated sequentially with mAb M4 in the presence of 10 mM LiCl. The soluble inositol phosphate was extracted with trichloroacetic acid (TCA) and applied to 1 ml of AG 1-X8 (formate form) ion exchange columns (Bio-Rad) pre-equilibrated with 0.1 M formic acid. After loading the samples, columns were washed with 10 ml of H_2O and 10 ml of 60 mM ammonium formate–5 mM sodium tetraborate, and elution was performed with increasing concentrations of ammonium formate (0.1–0.7 M) (Berridge *et al.*, 1983).

In vivo labeling and immunoprecipitation

Wild-type, PLC-γ2- (Takata *et al.*, 1995) and type 1/type 2/type 3 triple-deficient cells expressing murine MARCKS (1×10^7) were resuspended in 1 ml of phosphate-free RPMI 1640 supplemented with 10% dialyzed FCS. After 1 h incubation, cells were labeled with 1.0 mCi of [^{32}P]orthophosphate for 3 h at 37°C and washed twice in the same buffer. After mAb M4 stimulation (2 μg/ml), cells were chilled on ice for 30 min, and solubilized in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA) containing 50 mM NaF, 10 μM molybdate, 0.2 mM vanadate supplemented with protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM benzamidine hydrochloride, 10 μg/ml chymostatin, 0.1 mM TLCK, 0.1 mM TPCK, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml calpastatin I and 10 μg/ml pepstatin]. Insoluble material was removed by centrifugation at 13 000 *g* for 15 min. Cell lysates were incubated sequentially (1 h, 4°C for each incubation) with anti-MARCKS antibody and protein A–Sepharose. The immunoprecipitates were washed four times with lysis buffer and samples were separated in an 8% SDS–PAGE gel. Phosphorylation of transfected MARCKS was assessed by autoradiography. For immunoblotting, SDS–PAGE gels were transferred to nitrocellulose membranes (Amersham) and the filters were incubated with anti-MARCKS antibody. After washing, the filters were developed using horseradish peroxidase-conjugated anti-rabbit IgG antibody and enhanced chemiluminescence (ECL).

Binding assay for M1 muscarinic receptor expression

Transfected clones were assayed for expression of muscarinic receptor essentially as described (Goldsmith *et al.*, 1989). Briefly, intact cells (10^6 cells/sample) were incubated for 90 min with the muscarinic receptor antagonist [^{3}H]QNB (100 pM, 47 Ci/mmol, Amersham). All incubations were performed in duplicate, and background binding activity was determined in the presence of 10 μM atropine. Then cells were collected on a Whatman GF/B membrane, washed extensively and bound radioactivity was determined by liquid scintillation counting. For carbachol-induced calcium mobilization experiments, at least three independent clones with a similar expression level of M1 muscarinic receptor on each type of IP_3R-deficient DT40 mutant cell were examined.

NF-AT activity

Twenty four hours after transfection with 20 μg of NF-AT luciferase and 2 μg of pRL-CMV (Promega), 2×10^5 transfected cells were aliquoted into a 96-well plate and cultured in a final volume of 100 μl of RPMI 1640 medium. Cells were unstimulated or stimulated (3 μg/ml mAb M4) at 37°C in the growth medium. After 5 h stimulation, cells were lysed and luciferase activity was quantitated with Lumat LB 9501 (Berthold Japan) using the Dual-Luciferase™ Assay System (Promega). Luciferase activity was determined in triplicate for each experimental condition.

Flow cytometric analysis for apoptosis

For DNA content analysis, stimulated or unstimulated cells (1×10^6) were pelleted and resuspended in 0.7 ml of hypotonic DNA staining solution (50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100). Samples were kept at 4°C for 3 h, and subjected to analysis by FACScan (Becton Dickinson). Debris and doublets were excluded by appropriate gating.

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